# Comparison of lignin peroxidase, horseradish peroxidase and laccase in the oxidation of methoxybenzenes

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Lignin peroxidase oxidizes non-phenolic substrates by one electron to give aryl-cation-radical intermediates, which react further to give a variety of products. The present study investigated the possibility that other peroxidative and oxidative enzymes known to catalyse one-electron oxidations may also oxidize non-phenolics to cation-radical intermediates and that this ability is related to the redox potential of the substrate. Lignin peroxidase from the fungus *Phanerochaete chrysosporium*, horseradish peroxidase (HRP) and laccase from the fungus *Trametes versicolor* were chosen for investigation with methoxybenzenes as a homologous series of substrates. The twelve methoxybenzene congeners have known half-wave potentials that differ by as much as ~ 1 V. Lignin peroxidase oxidized the ten with the lowest half-wave potentials, whereas HRP oxidized the four lowest and laccase oxidized only 1,2,4,5-tetramethoxybenzene, the lowest. E.s.r. spectroscopy showed that this congener is oxidized to its cation radical by all three enzymes. Oxidation in each case gave the same products: 2,5-dimethoxy-*p*-benzoquinone and 4,5-dimethoxy-*o*-benzoquinone, in a 4:1 ratio, plus 2 mol of methanol for each 1 mol of substrate. Using HRP-catalysed oxidation, we showed that the quinone oxygen atoms are derived from water. We conclude that the three enzymes affect their substrates similarly, and that whether an aromatic compound is a substrate depends in large part on its redox potential. Furthermore, oxidized lignin peroxidase is clearly a stronger oxidant than oxidized HRP or laccase. Determination of the enzyme kinetic parameters for the methoxybenzene oxidations demonstrated further differences among the enzymes.

## INTRODUCTION

Growing evidence indicates that the lignin peroxidase of the fungus *Phanerochaete chrysosporium* plays a central role in the initial degradation of the complex aromatic polymer lignin [1–4]. Lignin peroxidase catalyses reactions not normally associated with other peroxidases, that is, oxidation of non-phenolic aromatic substrates. The plethora of products formed from its substrates have been explained as the consequence of multiple, substituent-dependent, reactions taking place after the initial formation of aromatic cation radicals [5–10]. The same products have been found in experiments with non-enzymatic one-electron oxidants [11,12].

The methoxybenzenes, of which there are 12 congeners, were particularly useful as substrates in elucidating the cation-radical mechanism of lignin peroxidase [7]. In the presence of $H_2O_2$, the enzyme oxidizes 10 of the 12 congeners, forming from several of them relatively stable cation radicals [7]. From the kinetics of radical formation, and results of product analyses, we concluded that oxidation of 1,4-dimethoxybenzene by lignin peroxidase results in demethoxylation to give *p*-benzoquinone and methanol as products. In studies with $H_2^{18}O$ we have shown that the quinone oxygen atoms come from water (P. J. Kersten, unpublished work).

The half-wave potentials ($E_{\frac{1}{2}}$) of the methoxybenzenes have been determined: these range from 0.81 V to 1.76 V versus a saturated calomel electrode [13]. This allows relationships to be examined between the redox potentials of the substrates and the ability of enzymes to oxidize them by one electron. Hammel *et al.* [5] reported that lignin peroxidase oxidizes polycyclic aromatics with ionizing potentials of approx. 7.5 eV or lower. Cavalieri & Rogan [14] reported that HRP will oxidize only those polycyclics with ionization potentials below 7.35 eV.

In the present study we compared lignin peroxidase from *P. chrysosporium*, horseradish peroxidase (HRP) and laccase from the ligninolytic fungus *Trametes* (syn. *Coriolus* or *Polyporus*) *versicolor* in their oxidation of methoxybenzenes. HRP was included because it has a catalytic cycle very similar to that of lignin peroxidase [15,16], and the redox potentials of HRP compound I/compound II and compound II/ferric couples have been well characterized [17]. In contrast with the peroxidases, laccase is a Blue Copper oxidase that catalyses the four-electron reduction of $O_2$ to $H_2O$ during its oxidation of phenolics, aromatic amines, ascorbate and metal cyanides [18]. Although some substrates are potential two-electron donors, the Blue oxidases are reduced in one-electron steps. This is due to the reduction mechanism in which Type 1 copper is the primary electron acceptor. The substrate radicals thus formed take part in further non-enzymic reactions [19,20]. The laccase from *T. versicolor* has Type 1 and Type 3 copper sites of particularly high redox potential in comparison with other Blue Copper proteins [21,22]. Furthermore, evidence has been presented recently that laccase, like lignin peroxidase, plays a role in lignin degradation by fungi [23], which makes additional information on its mode of action of interest to that area of research. A preliminary account of our results with lignin peroxidase and HRP has been presented [24].

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## MATERIALS AND METHODS

### Enzymes

Lignin peroxidase isoenzyme H8 was purified [25] from the extracellular broth of cultures of *Phanerochaete chrysosporium* [26]. It had a specific activity of 22 units·mg$^{-1}$ on the basis of the oxidation of veratryl alcohol at pH 3 [27]. Protein determination of purified H8 was based on an $\epsilon_{409}$ of 168 mM$^{-1}$·cm$^{-1}$ [15]. HRP (Type VI) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and used without further purification. The two chromatographic forms, A and B, of laccase from *Trametes versicolor* were prepared according to previously published methods [28,29]. Both laccase forms were carefully characterized by optical-absorption, c.d. and e.s.r. spectroscopy. These studies excluded the possibility of any contaminating peroxidases [22].

### Chemicals

Methoxybenzenes and 2,5-dimethoxy-*p*-benzoquinone were synthesized or purchased as previously described [7]. 4,5-Dimethoxy-*o*-benzoquinone was synthesized from catechol by the method of Wanzlick & Jahnke [30] [m.p. 224–227 °C (literature value 225–227 °C)].

### U.v.–visible studies

To test wheher a methoxybenzene congener was a substrate, as determined by u.v.–visible spectral analysis, enzyme reactions were run in 25 mM-sodium tartrate, pH 3.0, with a congener concentration of $2 \times 10^{-4}$ M and 10 μg of enzyme per ml of reaction mixture. With lignin peroxidase and HRP, the reactions were initiated with $4 \times 10^{-4}$ M-H$_2$O$_2$. To monitor the decay of the 450-nm chromophore produced during the oxidation of 1,2,4,5-tetramethoxybenzene, $1 \times 10^{-4}$ M-substrate and $2 \times 10^{-4}$ M-H$_2$O$_2$ were used. For the determination of enzyme kinetic parameters with 1,2,4,5-tetramethoxybenzene at 450 nm ($\epsilon_{450} = 9800$ M$^{-1}$·cm$^{-1}$), enzyme levels were used so that the initial velocities were much greater than the rate of cation-radical decay. For the kinetic studies with pentamethoxybenzene, oxidation was monitored at 300 nm; a $\Delta\epsilon_{300}$ of 10800 M$^{-1}$·cm$^{-1}$ was determined for this oxidation. Because the reactions of the peroxidases are bisubstrate, saturating H$_2$O$_2$ (0.5 mM) was used to determine the kinetic parameters for the methoxybenzenes. Reactions with laccase were air-saturated.

### E.s.r. measurements

E.s.r. measurements were performed as previously described [7], with reaction conditions the same as those described above for 'U.v.–visible studies'.

### Product identification and quantification

2,5-Dimethoxy-*p*-benzoquinone and 4,5-dimethoxy-*o*-benzoquinone produced from the oxidation of 1,2,4,5-tetramethoxybenzene were identified and quantified with a Gilson (Middleton, WI, U.S.A.) System 41 Gradient Analytical h.p.l.c. apparatus equipped with a Vydac (Hesperia, CA, U.S.A.) reverse-phase Ultrasphere ODS column. A methanol/water (1:4, v/v) solvent was used at 1 ml/min and the absorbance monitored at 282 nm. Reaction conditions were as described above under 'U.v.–visible studies', with a reaction time of 2 h. Authentic samples were used for standardization. Methanol was also quantified by using alcohol oxidase from the yeast *Pichia pastoris* (Sigma) and a YSI model 53 oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.) fitted with a Gilson single-port 1.5 ml reaction chamber. The pH of the HRP-catalysed reaction mixture was adjusted to ~ 7.2 by the addition of 2 M-sodium phosphate, pH 7.8, after a 1 h incubation, and the dissolved-O$_2$-levels monitored upon addition of 5 μg of catalase (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) and then 6 units of alcohol oxidase to 1.5 ml of the solution in the O$_2$-monitor chamber. Controls included the complete reaction mixture minus added H$_2$O$_2$.

### $^{18}$O-labelling studies

To test for $^{18}$O incorporation from H$_2$$^{18}$O, reaction mixtures contained 15 μg of HRP, $6 \times 10^{-4}$ M-1,2,4,5-tetramethoxybenzene, and $1.2 \times 10^{-3}$ M-H$_2$O$_2$ in 0.5 ml of tartrate buffer, pH 3.0, with either water or 10.6% excess $^{18}$O in water by dilution of 20% H$_2$$^{18}$O (Cambridge Isotope Laboratories, Woburn, MA, U.S.A.). A control reaction was also run in which 2,5-dimethoxy-*p*-benzoquinone was substituted for tetramethoxybenzene to test for non-enzymic exchange of the quinone oxygen atoms with water. After 12 min the reactions were stopped and the quinones reduced with Na$_2$S$_2$O$_4$. The reduced products were extracted into dichloromethane, the extracts dried over anhydrous Na$_2$SO$_4$, the solvent evaporated, and the residues acetylated with acetic anhydride/pyridine (1:1, v/v). Excess reagents were removed under vacuum, the residue redissolved in dichloromethane, and analysed by g.c./m.s. using a Finnigan MAT (San Jose, CA, U.S.A.) 4510 instrument with a 60 m, 0.2 μm-film-thickness (biscyanopropyl polysiloxane) SP-2340 fused-silica capillary column (Supelco, Bellefonte, PA, U.S.A.). The m/z values (relative intensity) at 70 eV for the enzymic reactions were as follows: in H$_2$O: 255 [$M^+$ (the molecular ion)+1, 0.15%], 254 ($M^+$, 9.1%), 212 ($M^+$ minus H$_2$C=C=O, 11%), 171 (6.7%), 170 ($M^+$ minus 2H$_2$C=C=O, 100%), 169 (2.0%), 156 (1.4%), 155 (44%); and in 10.6% H$_2$$^{18}$O: 256 (3.6%), 255 (1.2%), 254 (12%), 214 (3.5%), 213 (1.2%), 212 (13%), 174 (1.6%), 173 (2.6%), 172 (28%), 171 (10%), 170 (100%), 157 (12%), 156 (3.3%), 155 (39%). The $^{18}$O content of the 2,5-dimethoxy-*p*-benzoquinone product was determined by comparing the ion counts at m/z 170, 172, and 174.

## RESULTS

### U.v.–visible spectral studies

To investigate the possibility that methoxybenzenes are substrates for HRP and laccase, we looked for u.v.–visible spectral changes upon incubation of the enzymes with the substrates as described in the Materials and methods section. Of the 12 methoxybenzenes, only the 1,2,4,5-congener was oxidized by both enzymes (Table 1). The three congeners closest to 1,2,4,5-tetramethoxybenzene in half-wave potential also gave spectral changes with HRP, but these clearly occurred more slowly than the rapid oxidation of the 1,2,4,5-congener. In comparison, lignin peroxidase was able to oxidize 10 of the 12 methoxybenzene congeners under similar conditions.

The spectral changes with HRP/H$_2$O$_2$ and 1,2,4,5-tetramethoxybenzene are shown in Fig. 1; similar results were obtained with laccase and lignin peroxidase. Interestingly, this oxidation resulted in biphasic spectral changes, with the rapid formation of an intermediate having a well-defined peak at 450 nm, which then slowly disappeared with apparent first-order kinetics. Plots of log absorbance versus time indicated a half-life for the 450 nm chromophore of 9.7 min, and extrapolation to zero time indicated an absorption coefficient ($\epsilon_{450}$) of 9800 M$^{-1}$·cm$^{-1}$. Isosbestic points at 254 nm and 296 nm indicate that this intermediate is converted to final products without detectable accumulation of additional intermediates.

### E.s.r. characterizations

The cation radical of 1,2,4,5-tetramethoxybenzene was identified by e.s.r. after oxidation by HRP and by laccase. The

**Table 1. Oxidation of methoxybenzenes by lignin peroxidase, HRP and laccase**

| Compound | $E_\frac{1}{2}$ (V)* | Oxidation† by: | | |
|---|---|---|---|---|
| | | H8/$H_2O_2$ | HRP/$H_2O_2$ | Laccase/$O_2$ |
| Methoxybenzene | 1.76 | − | − | − |
| 1,3-Dimethoxybenzene | ND | − | − | − |
| 1,3,5-Trimethoxybenzene | 1.49 | ± | − | − |
| 1,2-Dimethoxybenzene | 1.45 | + | − | − |
| 1,2,3-Trimethoxybenzene | 1.42 | + | − | − |
| 1,4-Dimethoxybenzene | 1.34 | +‡ | − | − |
| 1,2,3,4-Tetramethoxybenzene | 1.25 | +‡ | − | − |
| Hexamethoxybenzene | 1.24 | +‡ | − | − |
| 1,2,4-Trimethoxybenzene | 1.12 | + | + | − |
| 1,2,3,5-Tetramethoxybenzene | 1.09 | + | + | − |
| Pentamethoxybenzene | 1.07 | + | + | − |
| 1,2,4,5-Tetramethoxybenzene | 0.81 | +‡ | +‡ | +‡ |

\* $E_\frac{1}{2}$ values are literature values [13] obtained by polarography with respect to a saturated calomel electrode; ND, not determined.

† A '+' indicates that u.v.–visible spectral changes were observed with enzyme and $H_2O_2$, whereas ' − ' indicates that they were not. A ' ± ' indicates that spectral changes were observed, but the enzyme was quickly inactivated.

‡ Cation radical detected directly by e.s.r.

hyperfine coupling parameters were as reported previously for the oxidation by lignin peroxidase [7] and as reported for the cation radical produced chemically [31]. The spectrum generated by oxidation with HRP/$H_2O_2$ is shown in Fig. 2 along with an inset showing the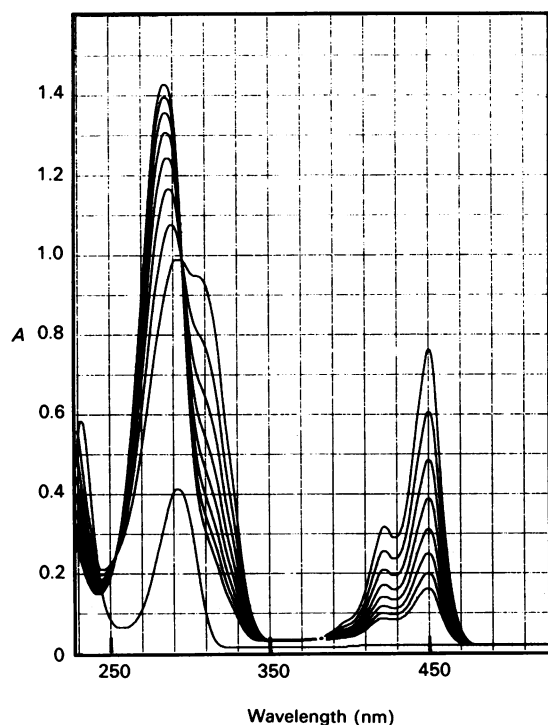 first-order decay of the cation radical; it has the same half-life as the 450 nm chromophore described above, indicating that the cation radical is the intermediate species detected in the u.v.–visible studies.

The oxidation of 1,3,5-trimethoxybenzene by lignin peroxidase H8 was unusual, not only because the enzyme was rapidly inactivated during the oxidation, but also because it was the only oxidation in the series for which a radical species could easily be detected by e.s.r. spectroscopy using 5,5-dimethyl-1-pyrroline N-oxide as spin-trap. The e.s.r. spectra were consistent with trapping of a carbon-centred radical in the absence of $O_2$ and an $O_2$-centred radical in air (results not shown). It is conceivable that inactivation of lignin peroxidase in air is linked to the production of peroxyl radicals, as has been suggested for horseradish peroxidase [32].

### Product analyses

On the basis of our previous results with lignin peroxidase and 1,4-dimethoxybenzene, an expected product from decay of the
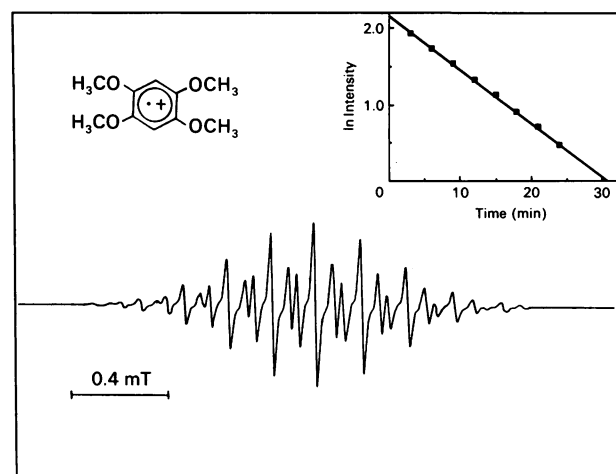


**Fig. 1. U.v.–visible spectral changes in 1,2,4,5-tetramethoxybenzene on incubation with HRP and $H_2O_2$**

Upon initiation of the reaction with $H_2O_2$, the spectrum of the starting material (290 nm $\lambda_{max}$, lowest spectrum) was quickly replaced with a new spectrum of $\lambda_{max}$ 290 nm (shoulder: 305 nm) and $\lambda_{max}$ 450 nm. The absorbance at 450 nm and at 305 nm then slowly decreased to give a new peak at 282 nm. The cycle time was 3.12 min. The reaction was run in 25 mM-sodium tartrate, pH 3, with $8 \times 10^{-5}$ M-1,2,4,5-tetramethoxybenzene, 10 $\mu$g of HRP/ml of reaction mixture and $3.3 \times 10^{-4}$ M-$H_2O_2$.



**Fig. 2. E.s.r. signal of the cation radical of 1,2,4,5-tetramethoxybenzene, obtained by oxidation with HRP and $H_2O_2$**

A similar spectrum was obtained with laccase with magnetic parameters as earlier determined with lignin peroxidase [7]. The inset shows the first-order decay of the signal, indicating a half life of 9.97 min. Intensity is given in arbitrary units.
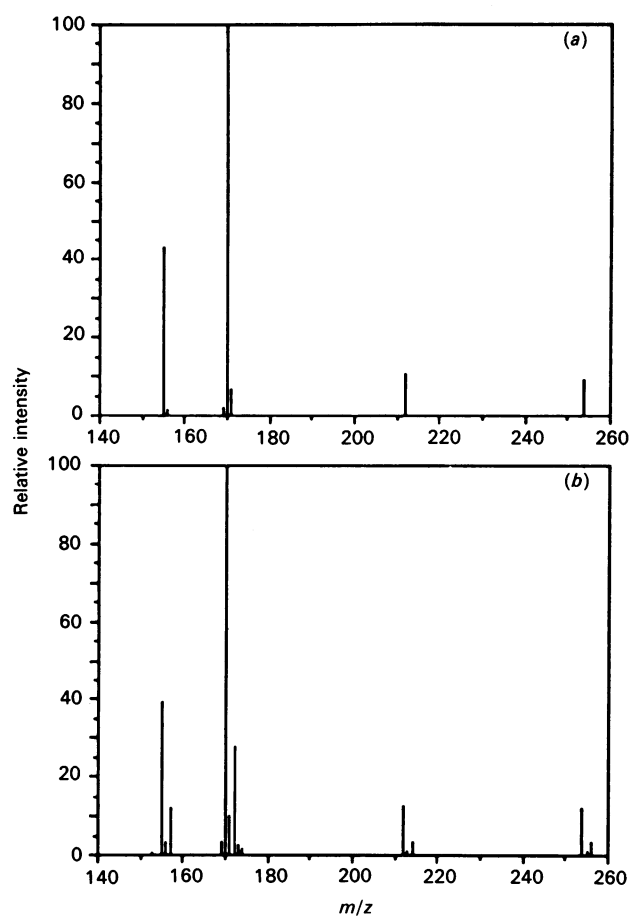
**Fig. 3. Mass-spectral analyses of $^{18}$O-labelled products**

2,5-Dimethoxy-$p$-benzoquinone from the oxidation of 1,2,4,5-tetramethoxybenzene by HRP/H$_2$O$_2$ was reduced and acetylated before analysis. Reactions were run in either natural-abundance H$_2$O ($a$) or enriched in $^{18}$O ($b$). A control with authentic 2,5-dimethoxy-$p$-benzoquinone incubated in an H$_2$$^{18}$O-reaction mixture showed no enrichment for $^{18}$O.
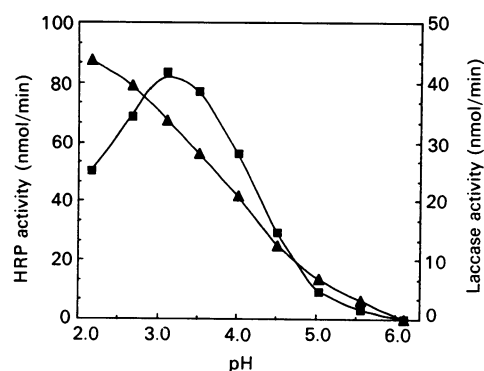
**Fig. 4. pH optimum for the oxidation of 1,2,4,5-tetramethoxybenzene by HRP and laccase A**

Reaction mixtures contained 7.5 mM-1,2,4,5-tetramethoxybenzene and either 50 ng of HRP (■) or 0.25 μg of laccase A (▲) in 1 ml of 50 mM-tartrate/citrate buffer. HRP-catalysed reactions were initiated with 0.5 mM-H$_2$O$_2$. Initial velocities were determined at 450 nm (see the Materials and methods section).
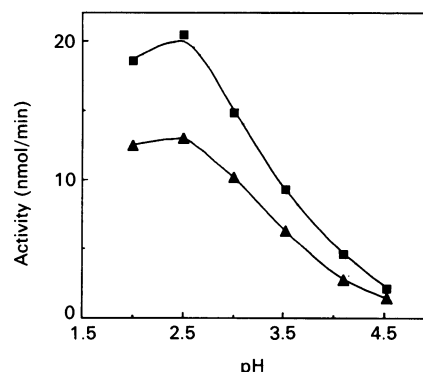
**Fig. 5. pH optimum for the oxidation of pentamethoxybenzene by lignin peroxidase and HRP**

Reaction mixtures contained 2 mM-pentamethoxybenzene, 0.5 mM-H$_2$O$_2$ and either 1.68 μg of purified H8 lignin peroxidase (■) or 10 μg HRP (▲) in 1 ml of 50 mM-tartrate buffer. In both cases, activity was less stable at pH values below 3.

**Table 2. Steady-state kinetic constants for the oxidation of 1,2,4,5-tetramethoxybenzene and pentamethoxybenzene**

Apparent kinetic parameters were determined spectroscopically as described in the Materials and methods section for reactions in 50 mM-sodium tartrate, pH 3.0. Oxidations of 1,2,4,5-tetramethoxybenzene were monitored by measuring the production of the cation radical; rates are therefore corrected for a two-electron oxidation.

| Enzyme | 1,2,4,5-Tetramethoxybenzene | | | Pentamethoxybenzene | | |
| --- | --- | --- | --- | --- | --- | --- |
| | $K_m$ (mM) | $k_{cat.}$ (s$^{-1}$) | $k_{cat.}/K_m$ (M$^{-1}\cdot$s$^{-1}$) | $K_m$ (mM) | $k_{cat.}$ (s$^{-1}$) | $k_{cat.}/K_m$ (M$^{-1}\cdot$s$^{-1}$) |
| H8 | 0.09 | 11 | $1.2 \times 10^5$ | 0.12 | 9.9 | $8.2 \times 10^4$ |
| HRP | 1.2 | 625 | $5.2 \times 10^5$ | > 7.5* | > 2.3* | 307* |
| Laccase A | 2.2 | 120 | $5.5 \times 10^4$ | −† | − | − |
| Laccase B | 1.5 | 124 | $8.3 \times 10^4$ | − | − | − |

* Non-saturating conditions were observed and therefore the second-order rate constant was determined by dividing the observed rate by enzyme and substrate concentrations.
† No oxidation detected.

cation radical of 1,2,4,5-tetramethoxybenzene would be 2,5-dimethoxy-$p$-benzoquinone [7]. In accord with this expectation, this quinone, as well as 4,5-dimethoxy-$o$-benzoquinone, was identified and quantified by h.p.l.c. The two products were formed in a ratio of approx. 4 ($p$-isomer):1 ($o$-isomer) and

accounted for all the ring-derived product within experimental error. Virtually the same qualitative and quantitative results were obtained with lignin peroxidase, HRP and laccase. Furthermore, an experiment with HRP and H$_2$$^{18}$O showed that the quinone oxygen atoms are derived from water (see Fig. 3). We also

determined that 2 mol $(1.9 \pm 0.2)$ of methanol are formed from each 1 mol of substrate oxidized.

## pH optima

As a prelude to steady-state kinetic studies, the pH optima for the oxidation of 1,2,4,5-tetramethoxybenzene by HRP and laccase (Fig. 4) and of pentamethoxybenzene by HRP and lignin peroxidase (Fig. 5) were examined. Surprisingly, in all cases a low pH optimum was observed for the initial velocities. A similar pH-optimum profile was obtained for laccase A assayed with a Clark-type oxygen electrode (results not shown).

## Steady-state kinetics

The data in Table 1 indicate substantial thermodynamic differences among the three enzymes. It was of interest therefore to compare kinetic parameters with substrates common to the enzymes, i.e. with methoxybenzenes of the lowest $E_{\frac{1}{2}}$ values. On the basis the pH-optima results above, and on previous investigations of the kinetic parameters of lignin peroxidase [15,33], the steady-state kinetics for the oxidation of 1,2,4,5-tetramethoxybenzene and pentamethoxybenzene were determined at pH 3 (Table 2). A saturating level of $H_2O_2$ (0.5 mM) was used with the peroxidases.

## DISCUSSION

Our results show that 1,2,4,5-tetramethoxybenzene is oxidized by HRP and laccase in the same way as it is by lignin peroxidase, namely via formation of the aryl cation radical. With all three enzymes, the cation radical degrades to the same products, indicating that the enzymes all have the same effect. Results with $H_2^{18}O$ and the HRP reaction showed that the quinone oxygen atoms are derived from water, as they are in the lignin peroxidase-catalysed oxidation of the 1,4-congener (P. J. Kersten, unpublished work). On the basis of these results,

we propose the sequence shown in Scheme 1 for the oxidation of 1,2,4,5-tetramethoxybenzene by the three enzymes.

There has been very little work to date on O-demethylation reactions catalysed by typical (non-ligninolytic) peroxidases such as HRP. The O-demethylation of 9-methoxyellipticine derivatives by HRP/$H_2O_2$ [34] resembles that of the methoxybenzenes [7; the present study] in that the methyl group is lost as methanol and the quinone oxygen atom of the quinone-imine products is derived from water. In the 9-methoxyellipticine case, however, the authors [34] proposed that the addition of water to intermediates occurs after two-electron oxidation (and loss of a proton) without the involvement of cation-radical intermediates.

With the homologous series of methoxybenzenes, oxidizability by the three enzymes correlated with $E_{\frac{1}{2}}$ of the compounds and not with the methoxyl substitution pattern (see Table 1). Because the $E_{\frac{1}{2}}$ values in Table 1 were obtained in acetonitrile with respect to a saturated calomel electrode [13], conclusions about the magnitude of these potentials in a different environment should be made with caution. We presume, however, that with the homologous series of substrates, solvent effects for the various congeners are similar and that comparisons within the series remain valid.

Our results with the methoxybenzenes indicate that all three enzymes oxidize their aromatic substrates by removing one electron from the aromatic nuclei. Apparently this is the first report of oxidation by laccase of aromatic nuclei bearing no hydroxy or amino group, although it is well known that lignin peroxidase oxidizes such substrates [1–5], and HRP oxidation of polycyclic aromatics has been reported [14]. Electron-donating groups such as hydroxy groups and amines, however, would be expected to facilitate oxidation by lowering the redox potential of the aromatic nuclei, which our results indicate is a critical parameter. In accord, all three enzymes oxidize a broad range of aromatic amines and phenols. This broad substrate specificity of
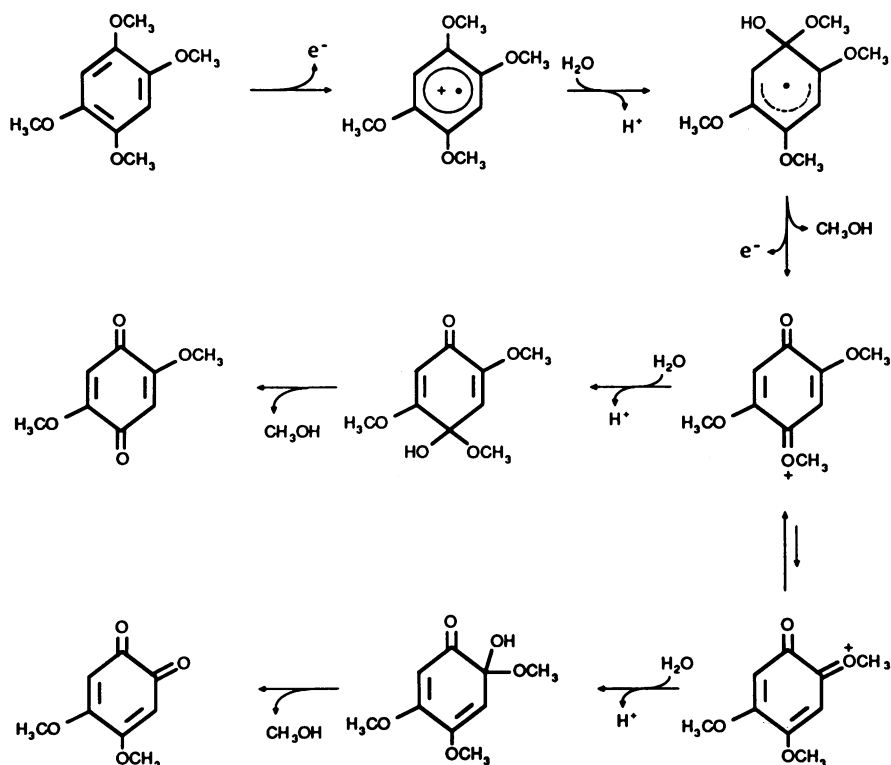
Scheme 1. Hypothetical scheme for the oxidation of 1,2,4,5-tetramethoxybenzene by lignin peroxidase/$H_2O_2$, HRP/$H_2O_2$ or laccase/$O_2$

the three enzymes suggests that they do not have specific binding sites.

The results also indicate that lignin peroxidase is a stronger oxidant than HRP, which in turn is stronger than laccase. The conclusion that oxidized HRP is a better oxidant than laccase is consistent with the published potentials for these enzymes. Using equilibria data from reactions coupled with the $K_2IrCl_6$–$K_3IrCl_6$ system, Hayashi & Yamasaki [17] determined redox potentials of $\sim 950$ mV for HRP compound I/compound II and compound II/ferric couples at slightly acidic pH values. From potentiometric studies using metal cyanides as electron mediators, Reinhammar [21] determined redox potentials of 785 and 782 mV for the Type 1 and Type 3 copper sites, respectively, of the laccase from *T. versicolor* at pH 5.5. The data of Table 1 would suggest higher redox values for these enzymes, probably owing to the lower pH and non-equilibrium conditions employed in our experiments. An oxidation potential more than 250 mV above that of oxidized HRP is suggested for oxidized lignin peroxidase at pH 3.

The kinetics for oxidation of the congeners of lowest redox potential demonstrate further differences in the enzymes. Notably, the $K_m$ values for 1,2,4,5-tetramethoxybenzene and pentamethoxybenzene with lignin peroxidase H8 are at least an order of magnitude lower than those observed with HRP and laccase. These $K_m$ values observed with H8, however, are in reasonable agreement with results obtained for veratryl alcohol, 1,4-dimethoxybenzene and the lignin model compound 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)-propane-1,3-diol, which represents the $\beta$-$O$-4 substructure of lignin [35]. In each case, a $K_m$ value between 50 $\mu$M and 150 $\mu$M was determined. Likewise, the $k_{cat.}$ values observed here are of the same order of magnitude.

1,2,4,5-Tetramethoxybenzene appears to be a better substrate for HRP than it is for lignin peroxidase. Despite the relatively high $K_m$ for this substrate with HRP, the $k_{cat.}$ value observed is about 60 times greater than that with H8, indicating a second-order rate constant ($k_{cat.}/K_m$) that is about four times greater for HRP than for H8. Likewise, the laccases have ten-fold higher $k_{cat.}$ values than lignin peroxidase with 1,2,4,5-tetramethoxybenzene; the $k_{cat.}/K_m$ values indicate that lignin peroxidase is only a slightly more efficient catalyst than laccase in this case. However, with pentamethoxybenzene a very different relationship is observed; lignin peroxidase is a much more efficient catalyst than HRP, and no activity is observed with the laccases.

The order of $k_{cat.}$ values for the three enzymes for the oxidation of the 1,2,4,5-congener is not the same as expected on the basis of the relative oxidation potentials of the enzymes. That is, $k_{cat.}$ (and $k_{cat.}/K_m$ as well) clearly depends on factors in addition to the oxidation potential of the enzymes. Such factors would be expected to include distance between interacting redox centres, orientation of substrate in or on the enzyme, and steric and electronic influences of neighbouring groups of the enzymes and substrates during oxidation [36].

Interestingly, the oxidation of 1,2,4,5-tetramethoxybenzene by all three enzymes was favoured at low pH. The low pH optima with the non-phenolic substrates suggest that oxidation is controlled by substrate-specific factors (e.g. stabilization of intermediates) that are favoured at low pH. The transient-state kinetics of lignin peroxidase compound I and compound II oxidation of veratryl alcohol likewise show a strong pH-dependence with faster oxidations at lower pH values [37].

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